More specifically, the present invention is directed to a method for producing an altered glycoforms of proteins having improved therapeutic values, *e.g.*, an antibody which has an enhanced antibody dependent cellular cytotoxicity (ADCC), in a host cell. The invention provides host cells which harbor a nucleic acid encoding the protein of interest, *e.g.*, an antibody, and at least one nucleic acid encoding a glycosyl transferase. Further, the present invention provides methods and protocols of culturing such host cells under conditions which permit the expression of said protein of interest, *e.g.*, the antibody having enhanced antibody dependent cellular cytotoxicity. Further, methods for isolating the so generated protein having an altered glycosylation pattern, *e.g.*, the antibody with enhanced antibody dependent cellular cytotoxicity, are described.

Furthermore, the present invention provides alternative glycoforms of proteins having improved therapeutic properties. The proteins of the invention include antibodies with an enhanced antibody-dependent cellular cytotoxicity (ADCC), which have been generated using the disclosed methods and host cells.

IV. BRIEF DESCRIPTION OF THE DRAWINGS

FIGURE 1 depicts structures of common types of N-linked oligosaccharides and nomenclature. M stands for mannose; Gn, N-acetylglucosamine (GlcNAc); G, galactose; Gn^b, bisecting GlcNAc; R, Asn-Gn-β1,4-Gn or Asn-Gn-β1,4-(α1,6-fucose)-Gn. R-M is called the oligosaccharide "core". The square brackets indicated that at least one Gn is linked to a G. the oligosaccharide nomenclature used in this work consists of enumerating the M and Gn residues attached to the R group, indicating the presence of a bisecting GlcNAc by including a Gn^b, and indicating if the oligosaccharide is galactosylated by including a G. The two types of tri-antennary oligosaccharides are differentiated by addint an apostrophe to the Gn_3 term.

FIGURE 2 depicts central reaction network on the N-linked glycosylation pathway. This set of Golgi-localized reactions determines the major types of structures into which N-linked oligosaccharides are normally classified. The enzyme catalyzing each reaction is shown and all reactions have been numbered. The reaction numbers are used to denote the kinetic parameters associated with each reaction (*see*, TABLE IV for example).

B. A Mathematical Model Of N-Linked Glycoform Biosynthesis

1. Overview

Metabolic engineering of N-linked oligosaccharide biosynthesis to produce novel glycoforms or glycoform distributions of a recombinant glycoprotein can potentially lead to an improved therapeutic performance of the glycoprotein product. Effect engineering of this pathway to maximize the fractions of beneficial glycoforms within the glycoform population of a target glycoprotein can be aided by a mathematical model of the N-linked glycosylation process. A mathematical model is presented here, whose main function is to calculate the expected qualitative trends in the N-linked oligosaccharide distribution resulting from changes in the levels of one or more enzymes involved in the network of enzyme-catalyzed reactions which accomplish N-linked oligosaccharide biosynthesis. It consists of mass balances for 33 different oligosaccharide species N-linked to a specific protein that is being transported through the different compartments of the Golgi complex. Values of the model parameters describing Chinese hamster ovary (CHO) cells were estimated from literature information. A basal set of kinetic parameters for the enzyme-catalyzed reactions acting on free oligosaccharide substrates was also obtained from the literature. The solution of the system for this basal set of parameters gave a glycoform distribution consisting mainly of complex-galactosylated oligosaccharides, distributed in structures with different numbers of antennae in a fashion similar to that observed for various recombinant proteins produced in CHO cells. Other simulations indicate that changes in the oligosaccharide distribution could easily result from alteration in glycoprotein productivity within the range currently attainable in industry. The overexpress of N-acetylglucosaminyltransferase III (GnT III) in CHO cells was simulated under different conditions to test the main function of the model. These simulations allow a comparison of different strategies, such as simultaneous overexpression of several enzymes or spatial relocation of enzymes, when trying to optimize a particular glycoform distribution.

The mathematical model disclosed herein consists of mass balances for 33 different oligosaccharide species N-linked to a specified protein that is being transported through the different compartments of the Golgi complex. These equations relate the oligosaccharide mole fractions to the amounts of the different enzymes, the kinetic

constants of the reactions, the distribution of enzymes in the different compartments, the half-life of the protein in the Golgi, the volume of the compartments, and the specific glycoprotein productivity. Values for the parameters in the model and their normal ranges can either be found in the literature or estimated from literature information. Some of the parameters are specific for each cell line. Those describing Chinese hamster ovary (CHO) cells were used here, since CHO cells are currently the most common host for the industrial production of therapeutic glycoproteins. Numerical simulations of the model with these values of the parameters gave glycoform distributions similar to those observed for some proteins produced in CHO cells.

One characteristic of the glycosylation pathway makes its modelling different from that of other biochemical pathways. Oligosaccharides have some degree of conformational flexibility and, through interactions with the polypeptide chain, certain conformations can be preferentially stabilized. Wyss and Gerhard, 1996, *Current Opinion Biotechnol.* 7:409-416. In addition, the polypeptide backbone around the glycosylation site may limit the access of the catalytic sites of the enzymes to the oligosaccharide. Shao and Wold, 1995, *Eur. J. Biochem.* 228:79-85. As a result, a particular glycosylation site can have its own set of values for the kinetic constants of the enzyme-catalyzed reactions. These values can be different from those of other glycosylation sites in the same or other proteins. The occurrence of this phenomenon can be inferred from the numerous examples where very different oligosaccharide distributions have been observed for different glycosylation sites of the same protein, even though all other system variables were identical for all sites during biosynthesis. Nevertheless, the range of values of the kinetic constants for oligosaccharides on some glycoproteins lies close to the corresponding range for soluble oligosaccharides (Do *et al.*, 1994, *J. Biol. Chem.* 269:23456-23464; Rao and Mendicino, 1978, *Biochemistry* 17:5635-5638; Gross *et al.*, 1990, *Anal. Biochem.* 186:127-134. Motivated by this observation, the constants for the latter were used as an initial approach to test the model and to study some aspects of its general behavior.

2. Physical Model

The N-linked glycosylation pathway consists of enzyme-catalyzed reactions which first attach a common oligosaccharide precursor to appropriate glycosylation sites in a polypeptide and then modify the linked oligosaccharides to produce a heterogenous set of glycoforms. Kornfeld and Kornfeld, 1985, *Annu. Rev. Biochem.* 54:631-664. Potential glycosylation sites are asparagine residues in the sequence Asn-X-Ser/Thr. The reactions take place in the endoplasmic reticulum (ER) and in the Golgi complex (Golgi) as proteins are transported through these cellular compartments *en route* to their final destinations. These destinations may be, for example, the ER or Golgi themselves, the plasma membrane, or the extracellular space.

The initial covalent attachment of the oligosaccharide precursor to the protein takes place during translocation of the latter into the lumen of the ER. Not all the translocated molecules acquire oligosaccharides in their potential glycosylation sites, and the fraction that does may vary between sites. The type of glycoform heterogeneity which thus results is called glycoform macro-heterogeneity. Shelikoff *et al.*, 1996, *Biotechnol. Bioeng.* 50:73-90. Once in the ER, the N-linked oligosaccharides are trimmed down by glycosidases which can sequentially remove three molecules of glucose and, sometimes, one of mannose. The glycoproteins are then transported to the Golgi where a different set of glycosidases and glycosyltransferases act on the N-linked oligosaccharides and lead to a diversity of structures. Such type of heterogeneity in the identity of the attached oligosaccharides is referred to as glycoform micro-heterogeneity.

A mathematical model of glycoform macro-heterogeneity has been published recently. Shelikoff *et al.*, 1996, *supra*. It incorporates different factors that determine the extent of the first transfer reaction of the pathway. In contrast, the model presented below is concerned with glycoform micro-heterogeneity. More specifically, it deals with a set of eight Golgi-localized enzymes which together determine the distribution of oligosaccharides into the following major structural classes: high mannose, hybrid, bi-, tri-, tri'- and tetra-antennary complex, bisected hybrid, and bisected bi-, tri-, tri'-, and tetra-antennary complex oligosaccharides (FIGURES 1 and 2). Thirty-three different oligosaccharide species are involved in 33 reactions catalyzed by these enzymes; including 5 high-mannose, 3 hybrid, 3 hybrid-galactosylated, 4 complex (bi-, tri-, tri',

and tetra-antennary complex), 4 complex-galactosylated, and the 14 bisected counterparts of the hybrid and complex oligosaccharides. The products of this set of reactions can be processed further in the Golgi through more transferase-catalyzed reactions that increase glycoform micro-heterogeneity.

The major elements of the physical model are: (a) the different Golgi compartments where the reactions take place and the transport of proteins between them, (b) the central network of enzyme-catalyzed reactions, and (c) the spatial distribution of these enzymes in the different Golgi compartments.

Golgi Compartments. The Golgi complex consists of a series of distinct, membrane-bounded compartments. Proteins destined to the extracellular space, plasma membrane, lysosomes, endosomes, or secretory storage fesicles are transported from the ER to the first Golgi compartment, the cis-Golgi network (CGN). From there, they travel in sequential order through the remaining compartments of the series; the cis-medial-, and trans-Golgi cisternae, which together comprise the Golgi stack; and then to the trans-Golgi network (TGN), the final sorting place. Rothman and Orci, 1992, *Nature* 355:409-415. There is some controversy about the number of cisternae in the Golgi stack, but in the present model only three are considered.

Proteins are transported between compartments by vesicles which bud off from the membrane of one compartment and fuse to the next in the series. Rothman and Wieland, 1996, *Science* 272:227-234. Secreted and plasma membrane proteins appear to go through the Golgi by a "bulk flow" mechanism. These proteins enter vesicles by default, *i.e.*, in the absence of specific transport or retention signals, and therefore at their bulk concentration in the donor compartment. Proteins which reside in the ER or Golgi require retention signals that allow them to be concentrated in the appropriate compartments. Such residency is not permanent and their relative concentration in a particular region is also aided by retrieval-vesicles that recognize transport signals in escaped proteins and return them to previous compartments.

For the physical model, four of the five Golgi compartments mentioned above are considered as a system of four reactors in series. The modelled compartments are the cis-, medial-, and trans-Golgi cisternae, and the TGN. This selection is based on immuno-electron microscopy studies that localize the enzymes included in the present

model to these compartments. Nilsson *et al.*, 1993, *J. Cell. Biol.* 120:5-13; Rabouille *et al.*, 1995, *J. Cell Science* 108:1617-1627. The chemical reactions catalyzed by these enzymes are described next.

Central Reaction-Network. The N-linked glycosylation pathway of mammalian cells has been deduced by a combination of *in vitro* and *in vivo* biosynthetic studies. Kornfeld and Kornfeld, 1985, *supra*; Schachter, 1986, *Biochem. Cell Biol.* 64:163-181. Although many enzymes participate in the pathway, a subset of them determines the distribution of oligosaccharides into 33 different species which together define the high mannose, hybrid, hybrid-bisected, complex and complex-bisected types. The network of reactions catalyzed by this subset is called the "central reaction network" (CRN). The CRN considered in the present model is depicted in FIGURE 2.

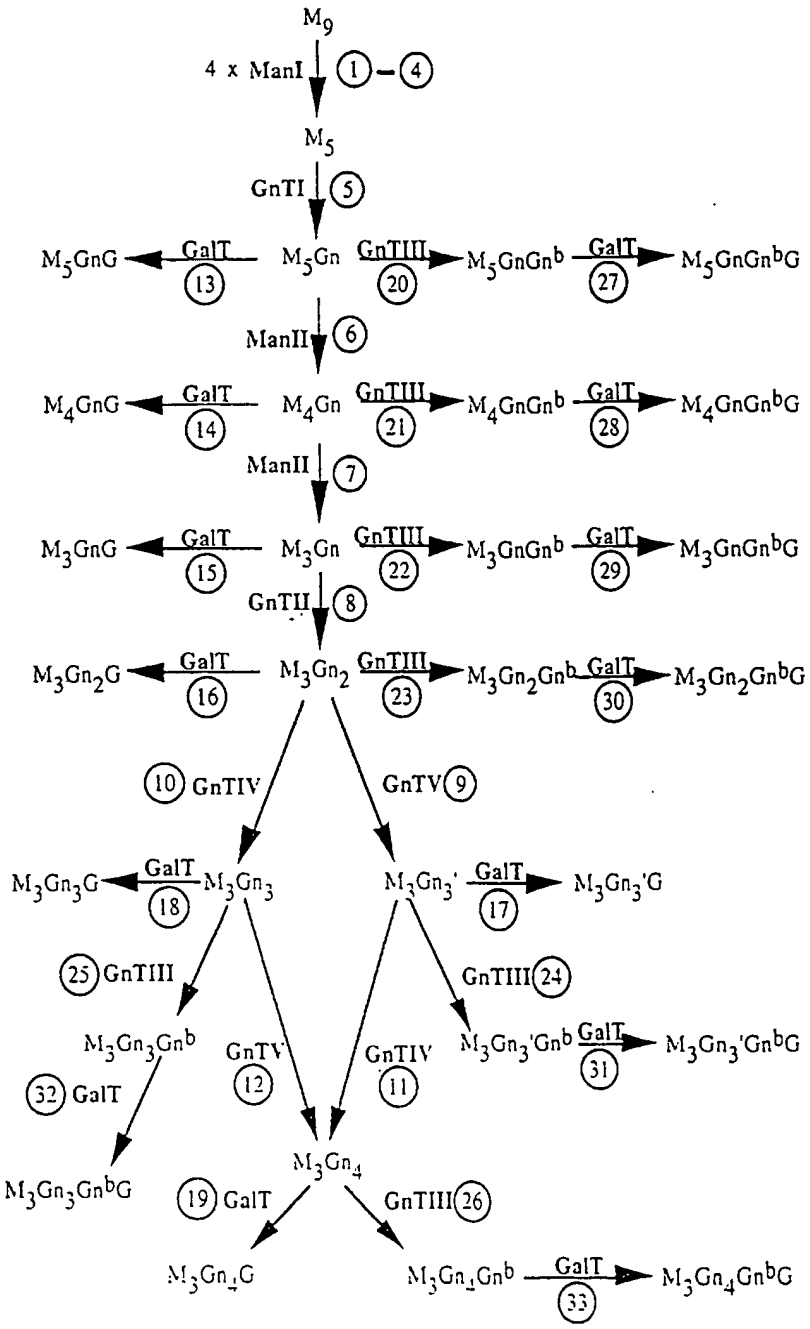
The first enzyme of the CRN is Golgi α1,2-mannosidase I (Man I), which can cleave α1,2-linked mannose residues from M_9-M_6 to finally produce M_5 (*see*, nomenclature in FIGURE 1), corresponding to reactions 1 to 4 in FIGURE 2. All eukaryotic cells have an α1,2-mannosidase in the ER that can also catalyze reaction 1. Therefore, the initial substrate for the Golgi CRN is a mixture of M_9 and M_8 oligosaccharides. Compounds M_9 to M_5 constitute the high-mannose class of N-linked oligosaccharides. The synthesis of hybrid and complex oligosaccharides then follows as described above.

An N-acetylglucosamine (glCNAc) can be transferred to the α1,3-mannose branch of M_5 by β1,2-N-acetylglucosaminyltransferase I (GnT I) to yield M_5Gn, the first hybrid oligosaccharide. M_5Gn is a substrate for α-mannosidase II (Man II), which catalyzes the removal of two mannose residues resulting in hybrids M_4Gn (Reaction 6) and M_3Gn (reaction 7). The free α1,6-mannose branch of M_3Gn is then available for extension by GnT II to produce M_3Gn_2, a complex bi-antennary oligosaccharide. M_3Gn_2 may be branched further by GnT IV or GnT V. GnT IV adds a GlcNAc in a β1,4-linkage to the α1,6-mannose branch, leading to the tri-antennary complex oligosaccharide M_3Gn_3. GnT V catalyzes a GlcNAc transfer in aβ1,6-linkage to the α1,3-mannose branch and produces the tri'=antennary complex oligosaccharide M_3Gn_3'. The tetra-antennary complex compound M_3Gn_4 can be synthesized both by GnT IV from M_3Gn_3' (reaction 11) and by GnT V from M_3Gn_3 (reaction 12).

All hybrid and complex oligosaccharides contain non-reducing-end GlcNAcs which may be extended by β1,4-galactosyltransferase (GalT, reaction 13 to 19). Once a galactose residue is transferred, the modified oligosaccharide is no longer a biosynthetic substrate for any of the remaining GnTs or for Man II. Schachter, 1986, *supra*. All of the branches in any complex oligosaccharide serve as substrates for GalT, but do so with different affinities. Pacquet *et al.*, 1984, *J. Biol. Chem.* 259:4716-4721. In the present model, these reactions are lumped together in single steps which remove the compound from the flux through reactions 1 to 12.

The reactions mentioned to this point take place in common industrial cell lines, such as CHO cells and baby hamster kidney (BHK) cells, used for the production of recombinant glycoproteins. Jenkins *et al.*, 1996, *Nature Bioltechnol.* 14:975-981. An additional set of reactions (20 to 33) is also important for determining the major classes of N-linked oligosaccharides in cell lines expressing GnT III. Examples of these cell lines are a glycosylation mutant of CHO cells named Lec 10 (Stanley and Campbell, 1984, *J. Biol. Chem.* 261:13370-13378) and rate myeloma (Y0) cells (Lifely *et al.*, 1995, *Glycobiology* 318:813-822. As indicated in reactions 20 to 26, GnT III can modify any non-galactosylated hybrid or complex oligosaccharide by transferring a GlcNAc residue in a β-1,4-linkage to the core mannose. The transferred residue is called a bisecting GlcNAc (Gn^b), and the products of these reactions are referred to as bisected oligosaccharides. GalT cannot extend the Gn^b residue, but it may modify all the other non-reducing-end GlcNAcs of any bisected oligosaccharide (reactions 27 to 33).

The final products of the CRN are usually modified further in the Golgi by the addition of sialic acids, poly-N-acetyllactosamine, fucose, N-acetylgalactosamine, sulphate, and α1,3-linked galactose. Wild type CHO cells only add sialic acids (in α2,3-linkages to galactose), fucose (α1,6-linked to the oligosaccharide core, *see*, R in FIGURE 1), and poly-N-acetyllactosamine (to various antennae but preferentially to that synthesized by GnT V). The addition of fucose to the core of oligosaccharides can take place at any point after reaction 5 of the CRN,b ut it is also blocked by the modifications that GalT or GnT III introduce. Core-fucosylated oligosaccharides can go through the rest of the CRN in the same way as their non-fucosylated counterparts,


M9
4 x ManI
1
4
M5
GnTI
5
M5GnG
GalT
13
M5Gn
GnTIII
20
M5GnGnb
GalT
27
M5GnGnbG
ManII
6
M4GnG
GalT
14
M4Gn
GnTIII
21
M4GnGnb
GalT
28
M4GnGnbG
ManII
7
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GalT
15
M3Gn
GnTIII
22
M3GnGnb
GalT
29
M3GnGnbG
GnTII
8
M3Gn2G
GalT
16
M3Gn2
GnTIII
23
M3Gn2Gnb
GalT
30
M3Gn2GnbG
10
GnTIV
GnTV
9
M3Gn3G
GalT
18
M3Gn3
M3Gn3'
GalT
17
M3Gn3'G
25
GnTIII
GnTIII
24
M3Gn3Gnb
GnTV
12
GnTIV
11
M3Gn3'Gnb
GalT
31
M3Gn3'GnbG
32
GalT
M3Gn3GnbG
M3Gn4
19
GalT
GnTIII
26
M3Gn4G
M3Gn4Gnb
GalT
33
M3Gn4GnbG

FIGURE 2